In the Specification

Please amend the title of the International Patent Application to read as follows:

~~UTILIZATION OF CELL FORCIBLY EXPRESSING TOLL-LIKE RECEPTOR~~
USE OF TOLL-LIKE RECEPTOR-EXPRESSING CELLS

Please add the following paragraph at page 1, above line 5 after the Title:

This application is a National Stage Application of International Application Number PCT/JP2004/002920, filed March 5, 2004; which claims priority to Japanese Application 2003-172132, filed June 17, 2003.

Please amend the specification at page 1 (Technical Field), line 6 as follows:

The present invention relates to use of cells ~~forced to express~~ expressing a Toll-like receptor.

Please amend the paragraph at page 1, lines 22-25 as follows:

Previous studies have reported the effect of lipopeptides on the expression activity of NF-κB in human TLR2-expressing CHO cells~~ that were forced to express human TLR2~~, as well as the effect of various CpG DNA motifs derived from pathogenic E. coli on cytokine (IL-8) yield in human TLR9-expressing HEK293 cells ~~that were forced to express human TLR9~~ (Non-Patent Documents 3 to 5).

Please amend the paragraphs beginning at page 2, line 17 through page 3, line 33 as follows:

In the development of functional food products, it is necessary to evaluate their ultimate effects on human, and for obtaining basic findings, investigation using experimental animals and animal cells is essential. To this end, the present invention focused on pigs as an

experimental animal, which has great potential utility as a human model system from aspects of organ transplantation and such, and is of great significance in food industry. In order to establish cells in which TLR9 is ~~forcibly~~ expressed for use in systems of assessing functional DNA, the present inventors decided to clone a swine TLR9 gene, and introduce the gene to ~~forcibly~~ express it in animal cells.

The present invention has been made in view of the above conditions. An objective of the present invention is to provide uses of TLR9-expressing cells ~~with forced TLR9 expression~~.

The present inventors cloned from the Peyer's patches of a swine intestinal tract a gene of Toll-like receptor 9, which is a receptor protein that recognizes the CpG DNA motif derived from pathogenic bacteria, and established animal cells (transfectants) in which swine TLR9 (sTLR9) is ~~forcibly~~ expressed. The presence of the sTLR9 protein in these animal cells is confirmed by generation and use of polyclonal antibodies against sTLR9. The sTLR9 transfectant was analyzed for its functionality on CpG DNA, and its application to systems for assessing LAB's DNA activity was sought.

Specifically, this was carried out according to the following (1) through (5):

(1) Total RNA was extracted from the Peyer's patch of a swine intestinal tract. Using primers prepared from highly conserved regions of human and mouse TLR9 genes, RT-PCR and RACE were performed to clone the swine TLR9 gene. The gene's full-length sequence was determined.

(2) The full-length amino acid sequence of swine TLR9 obtained from the genetic information was screened for antigenic determinant sites. The selected region was synthesized by peptide synthesis and used as an antigen for generating a swine TLR9 polyclonal antibody. Rabbits were immunized with the chemically synthesized antigen to generate polyclonal antibodies against swine TLR9 using standard techniques.

(3) HEK293T cells (human embryonic kidney cells) were transfected with the swine TLR9 gene to establish swine TLR9 gene-transfected cells (transfectant).

(4) SwineTLR9 expression in the HEK293T cells was confirmed by detecting swine TLR9 mRNA expression using RT-PCR. The expression of the swine TLR9 ~~membrane~~ protein was confirmed by immunostaining with a swine TLR9 polyclonal antibody using laser microscopy and flow cytometry.

(5) The reactivity of swine TLR9 against oligodeoxynucleotides (CpG2006 and CpG1826), which contain specific CpG DNA motifs that strongly stimulate human and mouse cells respectively, was analyzed.

The swine TLR9 gene, as revealed from the analysis result, is consisted of 3090 bases encoding 1029 amino acid residues (MW: 115.8 kDa). A 3145bp-long cDNA sequence comprising the swine TLR9 gene was determined. The amino acid sequence of swine TLR9 shows an extremely high homology to human TLR9 (82.9%) and a 74.9% homology to mouse TLR9, therefore swine TLR9 shows a relatively higher homology to human TLR9 than to mouse TLR9. The results of RT-PCR and immunostaining with a swine TLR9 polyclonal antibody revealed that the swine TLR9 protein was expressed as a membrane protein in the swine TLR9 transfectant, indicating successful creation of the swine TLR transfectant. Functional analysis conducted against CpG DNAs using this transfectant indicated that swine TLR9 has a higher reactivity with CpG2006 than with CpG1826. This analysis revealed that swine TLR9 can recognize a human-specific CpG DNA motif more effectively than a mouse-specific CpG DNA motif. Surprisingly, the results of comparing the levels of mRNA expression in various tissues by real-time PCR revealed that the mRNA expression in the Peyer's patches and mesenteric lymph nodes, which are tissues that have a central role in the intestinal tract immune system, was three or more times higher than in that of the spleen.

Please amend the paragraph at page 3, lines 35-36 as follows:

(a) contacting a test sample with a cell ~~forced to express~~ expressing an intestinal tract tissue-expressed Toll-like receptor; and

Please amend the paragraph at page 4, lines 18-19 as follows:

(b) contacting the extract with a cell ~~forced to express~~ expressing an intestinal tract tissue-expressed Toll-like receptor; and

Please amend the paragraph at page 5, lines 6-7 as follows:

[11] use of a cell ~~forced to express~~ expressing an intestinal tract tissue-expressed Toll-like receptor as a model intestinal immunocompetent cell;

Please amend the paragraph at page 5, lines 16-17 as follows:

[16] a cell ~~forced to express~~ expressing an intestinal tract tissue-expressed Toll-like receptor for use in the method of any one of [1] to [9];

Please amend the paragraph beginning at page 5, line 33 through page 7, line 2 as follows:

The intestinal tract, a boundary tissue between the interior and the exterior of a human body, is constantly exposed to external stimuli (e.g., microorganisms, such as bacteria and viruses, drugs, food additives, residual pesticides in food, and environmental pollutants).

Therefore, the intestinal tract serves not only to absorb orally ingested nutrients, but also as a first biological defense mechanism (first defense line) in the receipt, transportation, response and elimination of foreign substances (Mantis NJ. *et al.*, J. Immunol. 169 (2002) 1844-1851). Other defense mechanisms including lymphoid tissues and gut-associated lymphoid tissues (GALT) are present. GALT is made up of diffusive compositions and aggregative compositions. The diffusive compositions include intestinal intraepithelial lymphocytes and lymphocytes of the lamina propria mucosae, and the aggregative compositions include the Peyer's patches, lymphoid follicles and mesenteric lymph nodes (Spahn TW. *et al.*, Eur. J. Immunol. 32 (2002): 1109-1113). The Peyer's patches are covered with follicle-associated epithelium (FAE) and form dorm-like elevations in villus-free areas. The patches include a follicular area where germinal center B-cells are present and a parafollicular area where helper T-cells are present (Owen RL. Sem. Immunol. 11 (1999) 157-163). Membranous epithelial cells (M-cells), which are specialized epithelial cells that serve as the first line of defense in the local immune mechanism of intestinal tract, are dispersed in FAE. M-cells have a deep pocket that serves as a "tunnel" in which antigens are transported through the cytoplasm to the basolateral side, where they are presented to antigen-presenting cells, including ~~lymphocytes~~ B-cells, dendritic cells and macrophages. M-cells have also been found in the epithelial mucosa of trachea and reported to serve as an entry site for pathogens such as Bacillus tuberculosis (Teitelbaum R. *et al.*, Immunity. 10 (1999) 641-650). M-cells are also known to serve as an entry site for functional factors contained in food products, as well as for microorganisms and food antigens. Once taken up by M-cells, intestinal luminal antigens (especially macromolecules) are transported to the inside of Peyer's patches, where they come into contact with major histocompatibility complex (MHC) class II-positive antigen-presenting cells such as dendritic cells and macrophages (Kaneko K. *et al.*, J. Veterinary. Med. Sci. 61 (1999) 1175-1177; Gebert A. *et al.*, American J. Pathology. 154

(1999) 1573-1582; Jensen VB. *et al.*, Infection & Immunity. 66 (1988) 3758-3766; Penheiter KL. *et al.*, Mol. Microbiol. 24 (1997) 697-709; Debard N. *et al.*, Gastroenterology. 120 (2001) 1173-1182; Gebert A. *et al.*, Int. Rev. Cytology. 167 (1996) 91-159). Upon antigen stimulation, helper T-cells produce Fc receptors, antigen-binding factors (IBF), IL-2, IL-4, IL-5 and IL-6. T-cells and B-cells which are activated upon antigen presentation then start "homing": migrate via mesenteric lymph nodes into the thymus and are then transported via circulation into tissues under action, such as intestinal lamina propria mucosae, mammary gland, lacrymal gland, salivary gland and urogenital organs. B-cells then become plasma cells to produce IgA. The secretory IgA acts to eliminate viruses, bacteria, bacteriotoxins and allergens that enter the intestinal tract and other mucous tissues (Vaerman JP. *et al.*, Immunology. 54 (1985) 601-603; Machtinger S. and Moss R., J. Allergy. Clinical. Immunol. 77 (1986) 341-347; Mathewson JJ. *et al*, J. Infectious Diseases. 169 (1994) 614-617). Mesenteric lymph nodes develop beneath the Peyer's patches, where more lymphocytes, dendritic cells and macrophages are present across the Peyer's patches. The Peyer's patches and mesenteric lymph nodes thus play a central role in the intestinal tract immune system (immune system in the intestinal tract as above).

Please amend the paragraph at page 7, lines 3-9 as follows:

Meanwhile, the relation between intestinal mucosal epithelial cells and the uptake of FITC (fluorescein isothiocyanate)-labeled lipopolysaccharide (LPS), which is a known ligand of TLR4 and has been obtained from simian intestinal epithelium were analyzed using a TLR4 antibody and an IRAK antibody. The results indicated that LPS was taken up by the intestinal epithelial cells expressing TLR4 and IRAK and was transported to lamina propria mucosae (Imaeda H. *et al.*, Histochemical Cell Biology. 118 (2002) 381-388). This

observation suggests intestinal tract tissue-expressed TLRs, such as TLR9, are involved in the intestinal tract immune system.

Please amend the paragraph at page 7, lines 10-17 as follows:

In view of the foregoing knowledge, the present invention provides methods for assessing whether a test sample activates the intestinal tract immune system or not. In this method, the test sample is first contacted with cells (TLR transfectant) ~~that are forced to express~~ expressing an intestinal tract tissue-expressed TLR. In this step, the test sample is brought into contact with TLR on the surface of the transfectant. Activity of the TLR is then measured using signal transduction in the TLR transfectant as an indicator. In this assessment method, the test sample is judged to be activating the intestinal tract immune system if the TLR activity is increased as compared to that in a transfectant that has not been contacted with the test sample.

Please amend the paragraph at page 15, line 2 as follows:

Fig. 9 shows an alignment of TLR9 amino acid sequences (Continuation of Fig. ~~9~~8).

Please amend the paragraph at page 25, lines 3-12:

~~Cells with forced expression of swine~~ Swine Toll-like receptor 9 (TLR9)-expressing cells are prepared by cloning a TLR9 gene from swine intestinal Peyer's patches. Functional analysis on CpG DNAs using the above cells revealed that swine TLR9 shows a higher recognition ability for a human CpG DNA motif (CpG2006) than for a mouse-specific CpG

DNA motif (CpG1826). When the mRNA expression levels in various tissues are compared by the real-time PCR method, it is found out that the mRNA is expressed in Peyer's patches and mesenteric lymph nodes, which play important roles in the intestinal tract immune system, at a level thrice as much as in spleen or more. Thus, the cells ~~that are forced to express~~ expressing an intestinal tract tissue-expressed TLR (for example, TLR9) can be used to identify samples capable of activating the intestinal tract immune system.